Remarks

Regarding amendments to the specification

Regarding the requested amendment of paragraph [0001], the applicants requested an amendment of paragraph [0001] of the originally filed specification on October 1, 2002. That amendment of 10/1/2002 requested that paragraph [0001] include a claim to priority of application 60/326,331 and incorporate application 60/326,331 by reference into the specification of the present application.

Applicants, however, now understand that such an amendment was (and is) not permissible. This is because a statement of incorporation by reference cannot be added to an application after filing. Specifically, MPEP 201.11 (III. REFERENCE TO PRIOR APPLICATION(S)) states: "When a benefit claim is submitted after the filing of an application, the reference to the prior application cannot include an incorporation by reference statement of the prior application, unless an incorporation by reference statement of the prior application was presented upon filing of the application. See *Dart Indus. v. Banner*, 636 F.2d 684, 207 USPQ 273 (C.A.D.C. 1980)."

The present amendment of paragraph [0001] simply makes the text of paragraph [0001] identical to the originally filed text of paragraph [0001]. And this has the effect of canceling the improper amendment of paragraph [0001] of 10/1/2002.

Regarding the requested amendments of paragraphs [0147] and [0148], these amendments simply change the phrase "sample allele frequency" to "sample allele frequency data" by adding the word "data" once to each of the paragraphs. These amendments are made to correct obvious errors. These obvious errors are obvious to a person of ordinary skill from context (from reading paragraphs [0147] and [0148]). See especially the headings of each these paragraphs, which are from the Definitions Section [0056] of the application. These headings are "Sample allele frequency data" and "Genotype data/sample allele frequency data" respectively. See also, for example, mid paragraph [0163]. In mid paragraph [0163], the phrase "It is also possible to obtain sample allele frequency data" is correct and does not contain the obvious error as it does in [0147].

Regarding the requested amendment of paragraph [0249] and the addition of new paragraphs [0249.1] and [0249.2], these amendments do not add new matter to the application. The single sentence deleted from paragraph [0249] is restored as the first sentence of new paragraph [0249.1]. The single sentence includes an endnote, endnote 10. The remaining text in the two new paragraphs are taken from the references in endnote 10, Schuster, et. al. and Gyapay, et. al. (or refers to earlier related paragraphs in the application, [0143] and [0144]). The Schuster and Gyapay references are incorporated by reference into the application (see [0333]). And marked copies of the relevant pages (p. 100 Schuster, pp. 248-249 Gyapay) and passages are included herewith for the Examiner's convenience.

Conclusion

This Supplemental Amendment of the Specification follows an Amendment/Response of 5/30/2006 that responded to each point of rejection in the previous Office Action of 12/29/05. The applicants have requested the amendment of four existing paragraphs and have also requested the addition of two new paragraphs. No amendment adds prohibited new matter to the specification as explained in the Remarks Section above.

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Enclosures:

1) Gyapay, et. al. (1994) Nature Genetics, 7: pp. 248-249
2) Schuster, H. et al (1995) Nature Genetics, 13(1), p. 100

with an odds ratio of 1,000:1 or better. These maps, constructed with several tools based on the LINKAGE programme package, were compared to those using the same genotypes processed with the MultiMap algorithm[9], based on CRI-MAP[10]. Both sets of maps were essentially identical in order as well as distance. The main difference was observed with a few markers that were rejected by the GMS algorithm (Gene Mapping System)[11] because they could not be mapped to a region that was sufficiently precise whereas they were included in the comprehensive maps resulting from the MultiMap process. Refinement of the map and the increase in the number of markers has led to some modifications in the previous order of the markers. Four of these modifications concern markers previously ordered with odds ratios of greater than 1,000:1 and 14 concern markers positioned with lower probabilities.

Distribution of markers

Only one gap of over 20 cM remains on the map. The other gaps have been reduced in size. There are only 22 remaining gaps of over 10 cM, which represent 6 of the gaps which were over 20 cM on the previous map. A significant proportion of the markers from the H2 and H3 isochores (8 out of 62) were found to map to the distal end of the chromosomes and a number of the others are subtelomeric, as expected[12]. This indicates that markers from these GC-rich regions should permit a more dense coverage of numerous subtelomeric regions.

Correspondance between genetic and physical distance must await integration of genetic and physical maps. Genetic linkage maps integrating polymorphic markers from different sources including AFM markers from the first set of 814 have been established recently[9,13]. A more extended integration project using a different strategy and including new markers from the present map is in progress.

Conclusion

About 56% of our latest genetic linkage map of 3,690 cM is at a distance of 1 cM or less from one of its markers. In many cases, these distances can be covered by cloned DNA sequences[14]. Moreover, the isolation and mapping of 3,000 additional markers is in progress. This will increase the density of marker coverage and perhaps extend some of the chromosomal maps. This should accelerate considerably positional cloning of hereditary disease genes by facilitating the search for additional close genetic markers and candidate exons.

Methodology

Marker development. Marker development was carried out essentially as described[4]. DNA libraries were made from an *AluI* DNA digest of 46,XX human DNA (sized between 300–500 bp) and cloned in M13. The sequences of the templates from the $(CA)_n$ or $(GT)_n$ positive clones were used to define PCR primers. The synthesized primers were tested on four unrelated 46,XX individuals to obtain a first estimate of the polymorphism of the tested microsatellite markers. Markers with three or more alleles were first assigned to their chromosome and genotyped as described[4].

The H2 and H3 isochores were isolated by caesium sulphate density gradient centifugation of total human DNA in the presence of BAMD[8]. The $(AC)_n$ markers found in the fractions with the highest GC content were isolated by a single *AluI* digestion shotgun procedure as described[4].

Genotyping. Individuals from the eight CEPH families (102, 884, 1331, 1332, 1347, 1362, 1413 and 1416) were genotyped using standard procedures, as described[2]. PCR amplifications were performed in 50 µl reaction mixtures, containing 40 ng of genomic DNA, 50 pmol of each primer, 125 µM dNTPs, 10 mM Tris pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin and 1 U of *Taq* polymerase (Amersham). Amplifications were carried out using the "hot-start" procedure, in which the *Taq* polymerase is added to the reaction mixtures after a first denaturation step (5 min at 96 °C) after which 35 cycles of denaturation (94 °C for 40 s) and annealing (55 °C for 30 s) are performed. An elongation step (2 min at 72 °C) ends the process. For each DNA sample, 16 amplification products from different markers were ethanol-precipitated and loaded together into single lanes of 6% polyacrylamide-8M urea denaturing gels. After migration, the DNA was transferred from the gel to a Hybond N$^+$ nylon membrane (Amersham) by a contact blotting procedure[2]. The markers were then revealed by successive hybridizations with one of the PCR primers which was peroxidase-labelled by modification of the ECL procedure (Amersham) and exposed to autoradiographic X-ray films[2].

Map construction. Markers were assigned to chromosomes by pairwise linkage and possible genotyping errors were identified by comparisons between families of the pairwise recombination events between linked markers. After genotype corrections, markers from a chromosome-specific dataset were positioned on a framework consisting of the map of 814 markers[9] using a map construction algorithm. The order of markers in the framework and complete maps were determined with the GMS algorithm[11]. Briefly, recombination estimates for a preliminary, or trial order of the loci are used to divide the loci into subgroups of closely linked loci. Likelihoods are evaluated for different placements of subgroups, and for alternative orders of the loci within each subgroup. The best-supported order (i.e. the order with the greatest likelihood) is chosen as new trial order, and iterations are continued until convergence. Based on the best-supported order for the framework map, recombination fractions between adjacent markers were estimated with the LINKAGE programs[15]. Markers from this framework that underwent corrections since the 814-marker map were processed as new incoming markers. This led to a provisional order which was further reassessed as described[16]. Once the order remained unmodified after further computation, a search for double recombination events was undertaken. The maps were re-evaluated using the corrected genotypes until no further double recombination event could be eliminated.

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► Fig. 2 This map contains data for 2,066 $(AC)_n$ microsatellite markers, spanning the human genome. The chromosome maps showing the best-supported order of the markers and sex-average recombination fractions between adjacent markers are shown to scale. Most chromosomes are represented on several pages and data on the markers, such as the primer sequences, allele sizes and EMBL accession numbers, is given opposite the maps. Groups of markers for which the order cannot be resolved with odds > 1,000:1 are indicated by solid lines beside the names of the loci (on the right of each chromosome map). Rough locations are shown for selected reference markers from the CEPH database (version 6) on the left of the chromosome maps. The bars indicate regions of 1000:1 odds for positions of the reference markers based on location score analysis with respect to our maps. All Généthon markers described here have been submitted to GDB.

The number of alleles, the heterozygosity and the maximum and minimum allele sizes (size range) have been determined by observation of 28 unrelated individuals, namely the grandparents or the parents of the CEPH families 1331, 1362, 102, 1347, 1332, 1416, 1413 and 884.

The allele sizes indicated in the reference allele column are those observed in the mother of the CEPH family 1347 (individual 134702). Therefore, 134702 can be used as a reference for allele size determination. An asterisk indicates markers for which data on 134702 allele size was not available; in such cases, the size was derived from the cloned sequence.

Notes: (aa), L22371 (*D1S333*), Weber, J.L. (1993), unpublished; (ab), M87678 (*D11S866*)[17]; (ac), M98989[2]; (ad), X71445 (*NTRK1*), Greco, A. (1993), unpublished; (ba), X67747, (*D2S21-1*)[18]; (ca), L02085[2]; (da), L22427, Weber, J.L. (1993), unpublished; (db), X51852 (*UCP*)[19]; (dc), L00809 (*D4S192*)[20]; (dd), L09826 (*D4S826*)[20]; (de), L00804 (*D4S610*)[20]; (ea), L22411 (*D5S811*), Weber, J.L. (1993), unpublished; (fa), Z19340 (*D0S6908E*), Genexpress, (1992), unpublished; (ga), L22426 (*D7S803*), Weber, J.L. (1993), unpublished; (gb), J03764 (*PLANH1*)[21]; (ha), M94655[2]; (ia), M83639[22]; (ib), L10820 (*D9S125*), Kwiatkowski (1993), unpublished; (ka), X52579 (*D11S35*)[23]; (kb), L20022, Weber, J.L. (1993), unpublished; (la), M96789 (*GJA4*)[24]; (ma), M99151 (*D13S144*)[25]; (mb), M99142 (*D13S121*)[25]; (na), L04461, (*D14S99E*)[26]; (pa), L02208 (*D16S318*)[27]; (ra), M88273 (*D18S37*)[28]; (sa), Z11689 (*PSG-11*)[29]; (sb), M36089 (*XRCC1*)[30]; (sc), M89651 (ref. 31); (wa), X60693 (*DXS571*)[32].

the gene for parathyroid hormone related peptide (PTHrP). PTHrP is expressed by vascular cells, lowers blood pressure, and attenuates cell proliferation[11]. A recent gene disruption experiment resulted in homozygous mice with skeletal anomalies[12]. However, we have since ruled out the PTHrP gene as the gene responsible for this syndrome and are continuing our search. In our subjects a single gene is responsible for a 50 mm Hg elevation in blood pressure by age 50 years. Elucidation of this gene will likely give new insight into mechanisms of blood pressure elevation and may be of relevance to primary hypertension.

Methods

Family material. We visited the family described by Bilginturan *et al.*[2], which lives in a remote area on the north-eastern Black Sea coast of Turkey and examined 45 members after written, informed consent was obtained. Six additional members currently residing in Germany were examined 4 weeks later. Nine additional members were identified in a second visit to Turkey. Height and weight were obtained, as well as photographs of each individual's hands. Blood pressure and heart rate were measured by an oscillometric automated method. Venous blood was obtained for DNA extraction.

DNA-marker analysis. Genotyping was performed using the ABI PRISM Genotyping System, including the Linkage Mapping Set, PCR 9600 thermocyclers, ABI DNA Sequencers, GeneScan and Genotyper software from Applied Biosystems Division, Perkin-Elmer Corporation (ABD). The PCR primers contained in the Linkage Mapping set amplify dinucleotide repeat loci spaced at approximately 10 cM. The loci were selected from the human linkage map generated by Généthon[13] based on map position, heterozygosity and performance in routine PCR analysis. The markers in the Linkage Mapping Set are organized in 28 panels, with 9 to 17 primer pairs per panel whose products can be electrophoresed and detected in a single gel lane. Forward primers are labelled with either 6-FAM, HEX, or TET fluorescent dyes which can be distinguished due to their different spectral properties. PCR products related to specific markers are automatically identified in each lane by their molecular weight and fluorescent dye[14]. PCR reactions and electrophoresis were performed according to manufacturer's protocol. Electrophoresis and detection were done on both, an ABI 373 DNA Sequencer equipped with GeneScan 1.2 software and an ABI 377 DNA Sequencer equipped with GeneScan 2.0 software. Data were exported as a text file from Genotyper for subsequent linkage analysis.

Linkage analysis. Prior to linkage analysis, we performed extensive simulation studies using the SLINK simulation program[15]. Expected lod scores were determined for various subsamples of the pedigree under different genetic models for both, the trait and the marker locus, and at recombination fractions ranging from 0.01 to 0.1. The pedigree subsample chosen for the genome scan was 36 family members (21 affected, 15 nonaffected), and yielded an E-lod of +4.85 for a codominant marker locus having a heterozygosity of 0.75 and linked to the fully penetrant trait locus at a recombination rate of 0.05. Linkage analysis was performed using the LINKAGE package version 5.1 (ref. 16). For the trait locus we used a model of nearly complete penetrance (0.99) and low phenocopy rate (0.001), a disease allele frequency of 0.001 and absence of mutation. Children below age 10 years were typed as unknown. The codominant marker locus was individually modelled according to allele numbers and frequencies. Once significant linkage to chromosomal 12 was found, we switched to the full pedigree, including one loop (person IV.14, see Fig.1), and 9 children typed unknown for the trait phenotype and thus providing marker information only. This full system was used to calculate two-point and multi-point lod scores, using the FASTLINK package[17]. A multipoint map was constructed with the pedigree divided into three parts and with the four most relevant marker loci (allele numbers down-coded) using LINKMAP.

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